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Pepscan Mapping and Fusion-Related Properties of the Major Phosphatidylserine-Binding

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The binding of labeled phosphatidylserine (PS) to a collection of synthetic 15-mer peptides covering full-length glycoprotein G (G) of viral hemorrhagic septicemia virus (VHSV), a salmonid rhabdovirus, showed three dominant overlapping reactive peptides. This major PS-binding region was contained in a 28-mer peptide (p2; aa 82–109) with consecutive hydrophobic amino acid a–d heptad repeats (putative amphipathic α-helix) and 2 carboxy-terminal arginines. This 28-mer peptide showed a 10-fold higher apparent specific activity for PS binding than the 15-mer peptides. Binding to PS was also detected with virion-purified protein G but was not detected with other viral proteins. The highest apparent specific activity for PS binding was found with purified VHSV particles by both solid-phase and liquid assays. In contrast to the pH-independent PS binding to peptide p2, binding to virions was optimal at pH 5.6. PS binding to purified VHSV was greatly reduced by protease or detergent treatments that removed protein G, by treatment at pH 7.6, or by anti-p2 mouse antibodies at pH 5.6. The PS-binding region seems to be related to viral–host cell fusion since anti-p2 mouse antibodies inhibited VHSV-infected cell to cell fusion (fusion from within) and the pH profile of the VHSV-infected cell to cell fusion was similar to the pH profile of PS binding to VHSV. Comparative analysis showed that sequences similar to the major PS-binding domain of VHSV were also present in other fish rhabdoviruses and in rabies and vesicular stomatitis viruses.

INTRODUCTION

The homotrimeric glycoprotein G (G) of both mammalian (Gaudin *et al.,* 1993, 1992) and fish (Lecocq-Xhonneux *et al.,* 1994; Coll, 1995a) rhabdoviruses is responsible for its attachment to the receptor of the host cellular membranes and for its subsequent low-pH (≈5.5)-dependent membrane fusion activity (Rigaut *et al.,* 1991; Schlegel *et al.,* 1982; Superti *et al.,* 1984). Most probably the receptor-binding site and the so-called fusion peptide (and/or its surrounding sequences) would not be in the same envelope protein region as they are in influenza (Bizebard *et al.,* 1995; Bullough *et al.,* 1994; Carr and Kim, 1993) or in tick-borne encephalitis (Rey *et al.,* 1995) viruses, the only viral membrane proteins for which X-ray structure has been solved to date. However, the nature of the rhabdovirus receptor(s) is not yet clear, although previous studies have implicated host phospholipid binding as being important for the entry of rhabdoviruses into the cells they infect. Thus, phospholipids extracted from cellular membranes inhibited attachment and infection of rabies virus (Superti *et al.,* 1984) and of vesicular stomatitis virus (VSV) (Bailey *et al.,* 1984; Conti *et al.,* 1988; Mastromarino *et al.,* 1987; Schlegel *et al.,* 1982), and phosphatidylserine (PS) was the strongest inhibitor of VSV attachment at physiological pH (Schlegel *et al.,* 1983). Moreover, it is not yet known which region of the protein is implicated in the interaction with the host phospholipids (Gaudin *et al.,* 1995).

On the other hand, exposure of isolated rabies G trimers to low pH is known to induce conformational and size changes (Gaudin *et al.,* 1993) that expose hydrophobic inner regions to its surface (Bourhy *et al.,* 1993), similar to findings on the fusion peptide of the influenza virus (Bullough *et al.,* 1994; Carr and Kim, 1993). Also, the mapping of fusion-defective VSV mutants by insertion and site-directed mutagenesis have shown that although several regions distributed through the G molecule seem to alter fusion (Li *et al.,* 1993; Whitt *et al.,* 1990), mutations in the region from amino acid (aa) 123–137 could vary the optimum pH for fusion and were thus proposed for a putative internal fusion peptide (Zhang and Ghosh, 1994). Furthermore, the above-mentioned region (aa 58–221 for VSV and aa 102–179 for rabies virus) was labeled by hydrophobic photolabeling techniques under conditions favoring fusion (Gaudin *et al.,* 1995).

Because the lack of a vaccine against fish rhabdoviruses makes these the most damaging diseases of the international salmoniculture industry and of wild waters (Lorenzo *et al.,* 1995; Estepa *et al.,* 1994; Leong and Fryer, 1993; Lorenzen *et al.,* 1993), the study of fish rhabdovirus–cellular membrane interactions could be of importance in the design of alternative methods to fight these diseases. In the present study, we have characterized in detail the PS binding to the G protein of a fish rhabdovirus. This study focuses on the viral hemorrhagic septice-

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mia virus, VHSV (a fish rhabdovirus exhibiting G-mediated low-pH-dependent fusion) (Lecocq-Xhonneux *et al.,* 1994), and demonstrate, using synthetic peptides in solid-phase assays, that the major PS-binding region maps to aa residues ~80–110 of the G protein. Synthetic peptides from this region bound PS and antibodies against the dominant peptide inhibited both PS binding to VHSV and virus-induced cell to cell fusion. Comparative sequence studies showed that all other rhabdovirus G proteins analyzed have a similar predicted phospholipid binding domain that probably adopts an amphipathic α-helical structure.

MATERIALS AND METHODS

Purification of VHSV

The VHSV 07.71 isolated in France (LeBerre *et al.,* 1977) from rainbow trout *Onchorynchus mykiss* (Walbaum) was grown and/or assayed for infectivity in epithelial papillosum cyprine (EPC) cells. VHSV concentrated by ultracentrifugation or by polyethyleneglycol precipitation was layered on a 15 to 45% sucrose gradient in TNE (0.15 *M* Tris, 0.15 *M* NaCl, 1 m*M* EDTA, pH 7.6) and spun at 80,000 *g* for 270 min in a Beckman ultracentrifuge. The material banding at 31% sucrose contained the viral infectivity, whole virions, by electron microscopy, and all the viral proteins (purified VHSV). The material banding at 26% sucrose contained traces of viral infectivity, nucleocapsids, and the N and Nx viral proteins (Basurco and Coll, 1992). These fractions were further purified by ultracentrifugation over a 20% sucrose cushion.

Purified VHSV was treated with 1% Triton X-100, Tween 20, or CHAPS during 30 min at 20° and the G-free virions were isolated by ultracentrifugation over a 20% sucrose cushion at 80,000 *g* for 3 hr (Gaudin *et al.,* 1992). Purified VHSV (200 μg/100 μl) were treated with 1 mg/ml trypsin or V8 protease at 37°, pH 7.6, during 2 hr to digest the G. To label VHSV a [U-^{14}C]aa lysate (Amersham, Buckinghamshire, England) was added during the VHSV infection at 4 μCi/ml. The number of G molecules per virion (300 pmol of G/200 μg of VHSV) was calculated from densitometry valves of the autoradiograph of purified labeled VHSV separated by polyacrylamide gel electrophoresis (PAGE).

Synthetic peptides from the G sequence of VHSV

Synthesis of 15-mer peptides overlapping 5 aa and spanning the cDNA-derived aa sequence of G protein (Thiry *et al.,* 1991) of VHSV 07.71 was performed (Chiron Mimotopes, Victoria, Australia). The peptides were named by a number corresponding to the position of their middle aa (8th position) in the G, the first peptide being the p6, due to synthesis requirements (it contained 2 additional aa before the initial methionine). The peptides diluted in 5 m*M* HEPES, pH 7, were dried in 100 μl/well at a final concentration about 3000 pmol/well. Protein G peptides p2 (aa 82–109), p3 (aa 110–121), and p4 (aa 122–151) were from Clontech (Palo Alto, CA). Irrelevant peptides were pA (TWKEYNHNLQLDDGTC) and pB (PYRRDCVTTTVENED).

Analysis of the protein G of rhabdoviruses

The cDNA-derived G sequences of VHSV (Lorenzen *et al.,* 1993; Thiry *et al.,* 1991) and of other rhabdoviruses were studied with the ANTIGEN program from PCGene (Intelligenetics, Geneva, Switzerland). ΔG values $\geqslant$0.4 kcal/mol to transfer the aa sidechain from water to ethanol (Schulz and Schimer, 1984) defined the hydrophobic aa (F, Y, I, L, V, M, A + W, H, T) that were used to search for heptad-repeat sequences with the possibility of forming amphipathic helices (hydrophobic aa in a and d positions) with the program PSEARCH. The subsequence searched was (hydrophobic aa)XX(hydrophobic aa)XXX (X for any aa). The hydrophobic aa sequences of the predicted transmembrane and signal peptide regions were not considered (Coll, 1995b). Coiled coils (a–d positions of heptad repeats mainly occupied by aa F, Y, I, L, V, M, and A) were searched with the program COIL (Lupas *et al.,* 1991).

Preparation of anti-p2 mouse antibodies

Female BALB/c mice were injected with 20 μg of p2 peptide in Freund's complete adjuvant and then four monthly injections in Freund's incomplete adjuvant were carried out. To obtain about 40 ml of pooled diluted ascites containing antibodies, three p2-immunized mice were each intraperitoneally injected with 0.5–2 $\times$ 10^6 viable myeloma X63/Ag8653 cells (Coll, 1989). The anti-p2 was purified from ascitic fluid by chromatography over a p2-activated CH–Sepharose-4B column (Sigma Chemical Co., St. Louis, MO) with an 8-atom spacer. Specific antibodies were eluted with ethylenediamine, pH 12.5, neutralized, dialyzed, lyophilized, and reconstituted in phosphate-buffered saline, pH 7.4.

Purification of viral proteins

The G, N, M1, and M2 proteins were isolated in denatured form by preparative PAGE of purified VHSV in the presence of SDS and β-mercaptoethanol (Estepa *et al.,* 1991). Soluble G protein (Gs) was isolated by affinity chromatography over Sepharose–concanavalin A from VHSV-infected VHSV-free cell culture supernatants (Ruiz-Gonzalvo and Coll, 1993). The Gs contained 30% of the molecules as trimers as shown by ultracentrifugation and 70% as monomers of ≈60 kDa as shown by PAGE.

Solid-phase phospholipid binding assays

The labeled phospholipids, 53–55 mCi/mmol, L-3-phosphatidyl-[L-C3-^{14}C]serine, 1,2-dioleoyl (PS); L-3-

phosphatidylcholine, 1-palmitoyl-2-[1-^{14}C]linoleoyl (PC); or L-3-phosphatidylethanolamine, 1-palmitoyl-2-[1-^{14}C]-linoleoyl (PE) (Amersham, Buckinghamshire, England) were dissolved in organic solvents and dried in glass tubes. Once resuspended in phosphate/citrate buffer (Gaudin *et al.,* 1993) the mixture was sonicated at 4° for three 1-min periods to a size of 20 $\times$ 10^3 kDa as estimated by chromatography over Sepharose 4B (manuscript in preparation). Samples of 100 μl/well of viruses, viral proteins, or peptides were dried at 37° in 96-well plates (polystyrene from Costar/Nunc). Just prior to use, the coated plates were washed and 100 μl/well of labeled phospholipids in phosphate/citrate buffer was added. After 4 hr of incubation at 4°, the plates were washed three times with distilled water and incubated with 100 μl/well of 2% SDS, 50 m*M* ethylenediamine, pH 12.5, at 50° during 30 min. The extracts were transferred to 96-well polyethylene terephthalate plates (Wallac–Pharmacia), 100 μl of Hiload scintillation liquid (LKB, Loughtrough, England) was added per well, and they were mixed and counted on a 1450 Microbeta scintillation counter (Wallac, Turku, Finland, and Pharmacia Iberica). Backgrounds (maximum of 0.5 pmol of PS/well) were estimated by PS binding to empty polystyrene wells. The apparent PS binding was expressed as specific activity in picomoles of labeled PS bound to solid-phase per picomole of protein initially dried onto each well per 20 pmol of input labeled PS.

For the blotting experiments, 1000 pmol of peptides in 10 μl was spotted and dried onto an IPVH-20200 Immobilon-P transfer membrane (Millipore, Bedford, MA). The membrane was blocked with 1% defatted milk in phosphate/citrate buffer at pH 5.6, washed, and incubated with 10 ml of buffer containing 1500 pmol of PS at 20° during 4 hr. After washing and drying, the membrane was exposed for autoradiography.

Sucrose gradient ultracentrifugation of VHSV–phospholipid complexes

PS was incubated in the presence or absence of 200 μg (100 μl) of purified VHSV at 4° overnight in 1 ml of citrate/phosphate buffer at pH 7.6 or 5.6. Handling, incubation, and ultracentrifugation were carried out at 4° to minimize possible fusion of PS with the virus (Lenard, 1993). A sample containing PS (850,000 cpm) was incubated with VHSV or ^{14}C-labeled VHSV ([U-^{14}C]aa, Amersham) and applied to the top of a 3-ml discontinuous sucrose gradient (1 ml of 40% sucrose, plus 1 ml of 30% sucrose, plus 1 ml of 20% sucrose). The VHSV was separated by ultracentrifugation at 80,000 *g* during 4 hr at 4° in a Sorvall ultracentrifuge. After centrifugation, the gradients were collected in 150-μl fractions from the top of the tube and assayed for radioactivity.

Anti-p2 binding assay

Serial dilutions of the viral proteins or peptides dried onto the polystyrene solid phase were incubated with 0.3 μg/well of anti-p2 antibodies in phosphate/citrate buffer containing 0.5% albumin at pH 5.6. Detection of the amount of anti-p2 bound and color development was carried out with rabbit anti-mouse immunoglobulins conjugated to peroxidase and *o*-phenylenediamine (Sanz and Coll, 1992).

Neutralization/immunoperoxidase assay

About 10^3 $TCID_{50}$ per milliliter of VHSV 07.71 was incubated overnight at 4° with serial dilutions of anti-VHSV antibodies in 100 μl as described (Lorenzo *et al.,* 1995). Mixtures of virus/antibody were added to EPC monolayers in 96-well plates. After an adsorption time of 2 hr at 4°, the plates were washed and 100 μl/well of RPMI-1640, 2% fetal calf serum, and 20 m*M* HEPES was added. After an overnight incubation at 14°, the cultures were fixed for 10 min in cold methanol and dried. To detect the N antigen of VHSV, the MAb 2C9 diluted 1000-fold in dilution buffer (130 m*M* NaCl, 2 m*M* KCl, 8 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4, 0.24 m*M* merthiolate, 5 g of Tween 20/liter, 50 mg of phenol red/liter, pH 6.8) was added to the wells (100 μl/well) for 1 hr. Detection of the bound 2C9 was done with peroxidase-labeled anti-mouse IgG (Nordic, Tilburg, The Netherlands) and diaminobenzidine (DAB) (Sigma Chemical Co.) until development of brown foci in the controls. Brown foci were counted with an inverted microscope provided with a 10$\times$ ocular eye grid. Neutralization was expressed by the formula (number of VHSV N-antigen-positive foci/number of VHSV N-antigen-positive foci in control plates containing no antibodies) $\times$ 100. Neutralizing MAb C10 anti-VHSV obtained from Sanofi (Marnes-La-Coquette, France) was used as a positive control.

Fusion assay

To assay VHSV-induced fusion from within, EPC cells in 96-well plates (about 300,000 cells/well) were infected with 100 μl of cell culture medium containing VHSV 07.71 at a multiplicity of infection of 30 and incubated overnight at 14°. On parallel plates, antibodies were serially diluted in RPMI 1640 cell-culture medium (without bicarbonate) buffered with 20 m*M* HEPES and 20 m*M* MES (Sigma Chemical Co.) to pH 5.6 and incubated overnight at 14°. The next day, the EPC plates were washed and the serial dilutions of antibodies were added onto the EPC wells. After 30 min of incubation at 14°, the cultures were fixed during 10 min with cold methanol, stained with Giemsa, washed, and dried. About 200 nuclei per well were counted. Percentage of fusion was expressed by the formula (number of nuclei in syncitia/total number of nuclei) $\times$ 100. At 4° no VHSV-induced fusion from within could be detected by this assay as reported for VSV (Lenard, 1993).

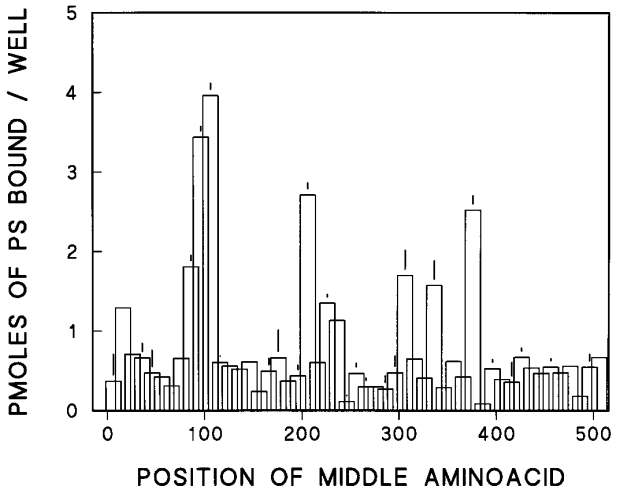


FIG. 1. PS binding to 15-mer overlapping G peptides from VHSV. PS (20 pmol per well) binding was at 4°, pH 5.6, during 4 hr. About 3000 pmol of peptides per well was used to coat the solid phase, depending on the peptide. The mean and standard deviations from two plates are represented.

RESULTS

PS binding to 15-mer G peptides

With a peptide load of about 300 pmol per well, a single peptide (p106, from aa 99 to 113) bound PS about three times more than the background level. The same unique peak was observed when the binding reaction was performed at pH 5.6 or at pH 7.6. No similar peak(s) could be detected with any of the G 51 peptides when incubated with labeled PE or PC (not shown).

Using plates coated with 10-fold the amount of peptides (Fig. 1), apparent maximal PS binding was again obtained with peptide p106 (about eightfold the background level). The extent of its apparent binding was not higher because of saturation of its PS binding at $>$100 pmol/well (Fig. 2). Some peptides with a PS binding $\geqslant$threefold the background were also obtained, like p96, p206, and p376, and others showed significant PS binding but gave values $\leqslant$threefold the background, like p16, p86, p226, p236, p306, and p336. PS binding by immobilon-blotted peptides, estimated by autoradiography, was positive only for p86, p96, p106 (most intense spot), p206, p226, p236, p336, and p376 (not shown). The PS binding of all the above-mentioned peptides was confirmed when individually tested by solid-phase PS binding in the range of 100–10000 pmol/well (not shown). The three contiguous overlapping peptides (p86, p96, and p106) show the highest PS-binding activity of the G pepscan (Table 1). No obvious similarities were apparent among all the highest PS-binding peptide sequences, except that they contain at least one positively charged aa (p106 contains two and p376 contains three). The absence of charged aa in the sequences of the putative signal peptide (aa 3–23) and the putative transmembrane domain (aa 474–494) might explain why they did not bind PS.

PS binding to solid-phase p2

Examination of the p106 sequence (aa 99 to 113) showed two arginines (aa 103 and 107) in their carboxy-terminal part (Table 1). The PS-binding G region (p86, p96, and p106) showed a computer-predicted α-helix from aa 82 to 102 contained in a longer hydrophobic aa (a–d hydrophobic aa) heptad-repeat region from aa 68 to 102. The presence of hydrophobic aa in the a–d positions of aa heptads would transform an α-helix into an amphipathic α-helix because of the clustering of the hydrophobic aa on one of the helix sides. The presence of proline at the 86 position would probably make it a bent α-helix. Therefore, peptide p2 (aa 82 to 109) containing both the putative amphipathic α-helix and the two arginines was synthesized.

The p2 showed about a 10-fold higher apparent specific PS-binding activity than the p106 (the highest PS-binding peptide of the 15-mer peptide pepscan) (Fig. 2). The PS binding to solid-phase p2, but not to p4 nor to p3, increased with time and was still slowly increasing

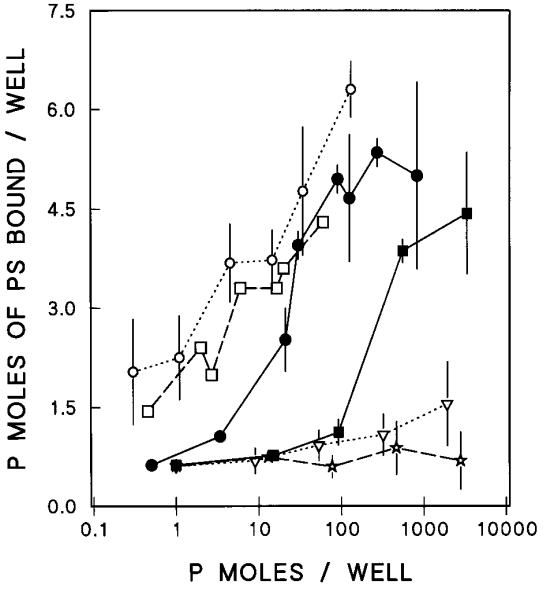
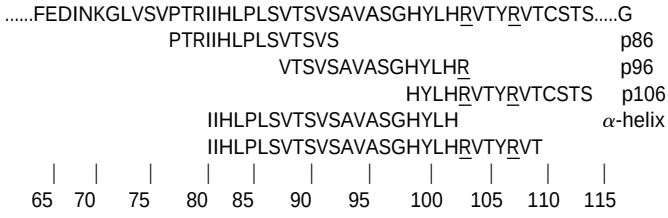


FIG. 2. Dependence of the PS binding on VHSV peptide, protein, or purified VHSV concentrations. PS binding (20 pmol/well) to different amounts of solid-phase peptides was performed at 4°, pH 5.6. (●) p2 (aa 82–109), (■) p106 (aa 99–113), (★) p4 (aa 122–151). Results obtained with p3 (aa 110–121) and the irrelevant peptides pA and pB were similar to p4. (□) native soluble G from VHSV-infected cell culture supernatants purified by affinity chromatography over concanavalin A–Sepharose (60 kDa), (△) electroeluted purified viral nucleoprotein N (38 kDa). The results obtained with electroeluted purified viral nucleoprotein Nx (34 kDa), electroeluted viral matrix protein M1 (24 kDa), electroeluted viral matrix protein M2 (20 kDa), and yeast recombinant nucleoprotein N3 (38 kDa) were similar to viral N. (○) purified VHSV. The averages and standard deviations of duplicates from different experiments are represented.

TABLE 1

Position of the p2 Peptide in the G Sequence of VHSV



Note. G, partial sequence of the G from VHSV 07.71 (Thiry *et al.,* 1991); p86 and p96, sequence of the 15-mer peptides from the pepscan that bind PS at the high peptide concentrations (Fig. 1); p106, sequence of the only 15-mer peptide from the pepscan that binds PS at the low peptide concentrations (Fig.1); α-Helix, predicted by the ANTIGEN program from the PCGene package; p2, designed synthetic peptide containing the α-helix plus the two-Arg (R)-containing sequence (RVTYRVT). Of the other 15-mer peptides of the pepscan that bound PS at the high peptide concentrations, only the p376 (^{369}YNRAQYKTMNNTWKS^{383}) was partially inside a–d hydrophobic aa heptad repeats from aa 377 to 398. The hydrophobic aa of the a–d hydrophobic heptad repeats are in bold. Numbers are the amino-terminal aa positions on the G including the signal peptide.

after 4 hr of incubation. The kinetics of PS binding to p2 were identical whether they were performed at pH 5.6 or at pH 7.6 and at 0 or at 20° (not shown). PS binding to solid-phase p2 performed in the presence of ⩾1000-fold molar excess of p2 showed background levels of binding but it was unaffected by the presence of a similar molar excess of p4 (not shown). The apparent PS binding increased with the concentration of p2 bound to the solid-phase to ⩾100 pmol of p2 per well and then reached a plateau. Only background levels of PS binding were obtained with p3, with p4, or with the irrelevant peptides pA or pB (Fig. 2). Similar profiles of the peptide concentration dependence on PS binding were obtained at pH 5.6 or 7.6 (not shown).

PS binding to purified VHSV proteins

The highest PS binding specific activity was obtained with affinity-purified Gs (10-fold higher than with denatured G protein purified from virions, Table 2 and results not shown), the only G protein preparation containing about 30% of trimers, suggesting that a special conformation is needed for optimal PS binding. Addition of ⩾1000 molar excess of cold PS over labeled PS reduced the PS binding of Gs to background levels (not shown). No significant PS binding could be detected when denatured nucleoprotein N, M1, or M2, purified from virions, were used to coat the plates (Fig. 2 and results not shown).

PS binding to solid-phase VHSV

Results obtained from different experiments showed that PS binding to solid-phase VHSV 07.71 was twofold higher at pH 5.6 than at pH 7.6. Addition of ⩾1000-fold molar excess of cold PS over labeled PS reduced the PS binding of solid-phase VHSV to background levels. The PS binding to solid-phase VHSV increased with time of incubation to reach a saturation value after 2–4 hr. Identical curves were obtained at 4 and at 20° (not shown). Figure 2 shows that the PS bound as a function of the concentration of VHSV expressed as its G content was the highest among all the VHSV proteins or peptides assayed. All the purified isolates of VHSV tested (F1, F2, 23.75, 798, 144, 471, 472, and 689) (Basurco and Coll, 1992), including one of infectious hematopoietic necrosis virus, another fish rhabdovirus (IHNV-Cedar) showed a similar extent of PS binding to solid-phase virus compared to VHSV 07.71 (not shown). Maximal apparent PS binding to solid-phase VHSV nucleocapsids (isolated by banding at 26% sucrose) showed only background levels in contrast to the PS binding obtained by its corresponding purified virions (isolated by banding at 31% sucrose) (not shown). Treatment of purified virions with either

TABLE 2

Apparent Specific Activity of PS Binding to Solid-Phase G Peptides, Gs, and VHSV

VHSV proteins	Assay	PS binding (pmol PS/pmol protein)	(*n*)
p106	Solid phase	0.01 ± 0.002	(4)
p2	Solid phase	0.10 ± 0.030	(6)
Gs	Solid phase	0.57 ± 0.370	(5)
VHSV	Solid phase	0.82 ± 0.790	(8)
VHSV	Solution	1.85 ± 0.380	(3)

Note. Picomoles of peptide or protein G, as calculated from the initial picomoles used to coat the solid phase. About 50% of the coated peptides or proteins remained in the wells after the binding assay was finished, as estimated by measuring the remaining proteins with the Bradford reagent (Bio-Rad, Richmond, VA). All determinations were done at pH 5.6, 4°, after 4 hr of incubation. Calculations were made in the lineal range of concentrations. Averages and standard deviations from *n* determinations are represented. p106 and p2, peptides from G according to Table 1; Gs, soluble G purified by affinity chromatography from VHSV-infected cell supernatants with about 30% of its molecules present as trimers; VHSV, whole virus purified by ultracentrifugation. Picomoles of G in the purified virions was estimated by autoradiography of PAGE of labeled purified VHSV.

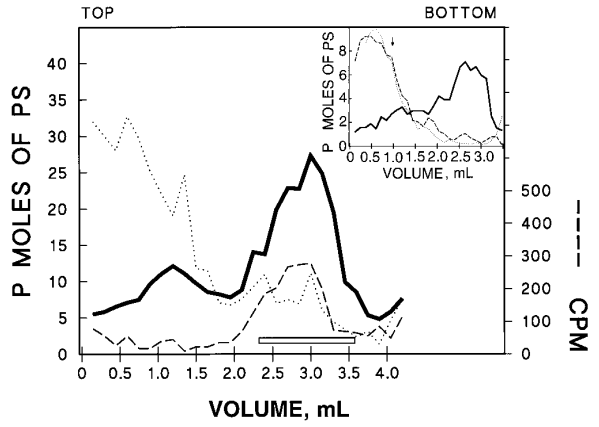


FIG. 3. PS binding to purified VHSV in solution. Purified VHSV (200 μg of VHSV containing about 300 pmol of G) and 1000 pmol of labeled PS were incubated overnight in 1 ml of buffer at pH 7.6 or 5.6 at 4° to inhibit fusion. Then they were ultracentrifuged in sucrose discontinuous gradients (from bottom to top, 1 ml of 40% sucrose + 1 ml of 30% sucrose + 1 ml of 20% sucrose + 1 ml of PS+VHSV mixtures) either at pH 5.6 or at pH 7.6 at 80,000 *g* for 4 hr. Fractions of 150 μl were collected from the top and samples of 75 μl were counted. The horizontal open bar shows the fractions which showed VHSV infectivity. Thick line, distribution of picomoles of PS in PS+VHSV incubated and centrifuged at pH 5.6; dotted line, distribution of picomoles of PS in PS+VHSV incubated and centrifuged at pH 7.6; dashed line, distribution of ^{14}C counts per minute (cpm) in labeled VHSV incubated and centrifuged at pH 5.6 (at pH 7.6 results were similar). The inset shows a similar experiment, thick line, PS+VHSV incubated and centrifuged at pH 5.6; dotted line, PS+CHAPS-treated VHSV incubated and centrifuged at pH 5.6; dashed line, PS+trypsin-treated VHSV incubated and centrifuged at pH 5.6 (results were similar when using protease V8). Vertical arrow indicates the banding position of catalase (240 kDa) when centrifuged under the same experimental conditions.

Tween or Triton to separate the G from the virions also reduced the PS-binding capacity of the solid-phase pelletable material to near background levels (not shown).

PS binding to VHSV in solution

Under the conditions employed and from pH 5.6 to 7.6, in the presence or in the absence of phospholipids, purified VHSV consistently banded between fractions at 31–35% of sucrose in different experiments as determined by ultracentrifugation of purified radioactively labeled VHSV. These results were confirmed by VHSV infectivity assays (representative results are shown in Fig. 3). Under the same conditions and between pH 5.6 and 7.6, any sonicated labeled phospholipids remained in the top of the centrifugation tube even after ultracentrifugation for 20 hr (not shown).

However, purified VHSV incubated with labeled PS and ultracentrifuged through a sucrose gradient at pH 5.6 showed comigration of VHSV infectivity and labeled PS. In contrast, at pH 7.6, 90% of the PS remained in the top of the tube, almost as if there was no VHSV present (Fig. 3). To minimize possible PS fusion to the VHSV, the experiments were performed at 4° and hence the observed interaction is most probably due only to PS binding. Treatment of purified VHSV with trypsin or protease V8 or with CHAPS abolished its PS-binding activity (Fig. 3, inset). About 80% of the labeled PS recovered in the sucrose gradient after ultracentrifugation at pH 5.6 comigrated with VHSV, suggesting that no saturation of the PS-binding sites of VHSV was obtained. In contrast, the opposite results (20–30%) were obtained with both PC or PE, probably as a result of lower affinity and/or saturation (not shown).

Partial denaturation of the virion after the solid-phase coating could explain why the amount of PS bound to solid-phase VHSV at pH 7.6 (50% of the amount bound at pH 5.6) is higher than the amount of PS bound to liquid-phase VHSV at pH 5.6 ($\leqslant$10% of the amount bound at pH 7.6) (Fig. 3).

When the peak of PS radioactivity from gradients containing PS+VHSV at pH 5.6 (from fractions 2.5 to 3.5 ml) was pooled and ultracentrifuged over a sucrose gradient at pH 7.6, banding of the radioactivity at 30–34% of sucrose was again obtained (not shown), demonstrating a high stability of the PS associated to VHSV.

Comparison of apparent specific activity of PS binding

Table 2 shows a comparison of the levels of apparent PS-binding specific activity expressed as picomoles of PS/picomole of peptides or proteins after several independent measurements of p106, p2, Gs, and VHSV. Thus, while p2 shows a 10-fold increase in PS-binding specific activity compared to p106, Gs and VHSV show a 5- and 8-fold increase, respectively, compared to p2. Estimates of the PS-binding specific activity of purified VHSV in solution show another 2-fold increase, to reach a maximal apparent binding of about 1.85 pmol of PS/pmol of G protein. However, since these measurements were made at values below saturation of PS binding the above-mentioned measurements are only valid as relative estimates.

Anti-p2 effects on PS binding, viral infectivity, and virus-induced fusion

To study the possibility of blocking PS binding to VHSV with anti-p2 antibodies, purified VHSV was first incubated with anti-p2 antibodies at pH 5.6 prior to the addition of PS. Inhibition of PS-binding to VHSV in solution by previous incubation of VHSV with anti-p2 varied from 45.4 to 70.3% (Fig. 4).

To ascertain whether the above-described PS-binding is really critical to the VHSV life cycle, we conducted a series of experiments to test if p2-specific antibodies would bind to virions, inhibit viral infectivity, or inhibit viral-induced fusion. By using ELISA, we first showed that anti-p2 antibodies bound to solid-phase VHSV to a greater extent at pH 5.6 than at pH 7.6, suggesting VHSV

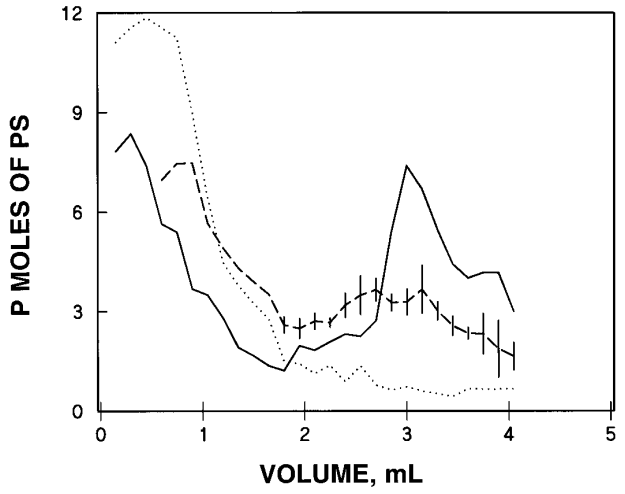


FIG. 4. Anti-p2 inhibition of PS binding to purified VHSV in solution. Purified VHSV (40 μg containing about 60 pmol of G) and 10 μg of anti-p2 antibodies were incubated for 1 hr at 20° in 150 μl of buffer at pH 5.6. The mixture was then cooled to 4°, 150 pmol of labeled PS in 500 μl of the same buffer were added, and the mixture was further incubated at 4° (to minimize possible fusion between PS and VHSV) for 2 hr. Ultracentrifugation of the mixtures was as described. Dotted line, PS; solid line, VHSV+PS; dashed line, VHSV+anti-p2+PS (average and standard deviations of duplicates are represented). A similar amount of total labeled PS was recovered (as calculated by addition of the label in each fraction) in each of the three cases.

must undergo a conformational change for the reaction to occur (Fig. 5A). Figure 5B shows that anti-p2 antibodies incubated with VHSV at pH 5.6 or at pH 7.6 were unable to neutralize VHSV in contrast to parallel control experiments which showed that VHSV was neutralized by commercial neutralizing MAb C10.

Similar experiments carried out by fusion assays showed that, whereas anti-p2 antibodies were capable of inhibiting fusion induced by the VHSV infection, anti-N MAb 2C9 or unspecific mice IgG1 + IgG_{2a} were not (Fig. 5C). Furthermore, the pH profile of PS binding by solid-phase VHSV was very similar to the pH profile of VHSV induced fusion either from within or from without (Fig. 6).

Comparative studies of the p2 sequence with other rhabdoviral G sequences

To search for the p2-like region in other rhabdoviruses, we first defined it as belonging to an amino-terminal part of the G with $\geqslant$three contiguous a–d hydrophobic heptad repeats containing an hydrophobic aa in position a or d. Heptad repeats such as those (not necessarily related to coiled-coil formation) suggesting amphipatic α-helices could be found in two to three positions in the G molecule in VHSV as well as in rabies virus and in vesiculoviruses (Coll, 1995b). The newly defined heptad repeats were found between aa 68 and 101, 288 and 319, and 377 and 400 for VHSV; between aa 99 and 119 and 327 and 359 for IHNV; between aa 140 and 164 and 330 and 360 for all rabies strains examined; between aa 134 and 161 and 332 and 356 for VSV-NJ; and between aa 134 and 161 and 328 and 369 for VSV-Ind. All these amino-terminal heptad repeat sequences were also followed by a short 5–7 aa sequence containing at least 1 positively charged aa (Table 3). Most of the positively charged aa were R as in VHSV, but K and H were also found in other rhabdoviruses. The new heptad-repeat regions detected showed a high degree of sequence conservation among rhabdoviruses belonging to the same family but were unrelated when members of different families were compared (Table 3).

DISCUSSION

The main PS binding domain of the G of VHSV has been identified using pepscan, synthetic peptides, isolated G, and purified VHSV solid-phase PS-binding assays. This is the first identification of a phospholipid-binding domain in any rhabdovirus and it also extends the well-known phospholipid interactions of mammalian rhabdoviruses (VSV and rabies) to fish rhabdoviruses.

The highest PS-binding peptide (p106, aa 99 to 113) was at the carboxy-terminal part of a predicted α-helix (aa 82–102) with a–d hydrophobic aa heptad repeats including aa W, H, and T, and a P (aa 86). This was the main PS-binding region as shown by two other dominant PS-binding peptides overlapping with p106 (p86, aa 79–93, and p96, aa 89–103) (Fig. 1). Its PS binding was further confirmed by blotting/autoradiography and by ELISA of the individual peptides. All these results led to the design of peptide p2 (Table 1) containing both the region of the heptad repeat with a putative amphipathic α-helix and a short aa stretch with two arginines (R, 103 and 107). Peptide p2 showed an $\sim$10-fold increased specific activity of PS binding compared to p106 (Fig. 2 and Table 2). The relevance of PS binding to solid-phase p2 to the possible PS binding to VHSV in solution was suggested by the capacity of a p2-affinity-purified anti-p2 polyclonal antibody to recognize both p2 and VHSV. The low molar ratio between the bound PS and the solid-phase p2 suggested that the dehydrated peptides in the solid phase would be unlikely to assume the conformation present in the native G molecule (Table 2). However, under carefully controlled experimental conditions, anti-p2 was also capable of inhibiting PS binding to VHSV in solution (Fig. 4).

PS binding to solid-phase p2 was independent of pH in contrast to PS binding to solid phase VHSV (Coll, 1995c). Most probably, pH 5.6 is needed for conformational changes in VHSV to increase its PS binding both in solid phase and in solution (Fig. 3). Confirming these data, the reaction of VHSV with anti-p2 by ELISA was also higher at pH 5.6 than at pH 7.6 (Fig. 5A).

Although the apparent specific activities of the PS binding of p2 (Fig. 2) or of native Gs (Fig. 2) are higher

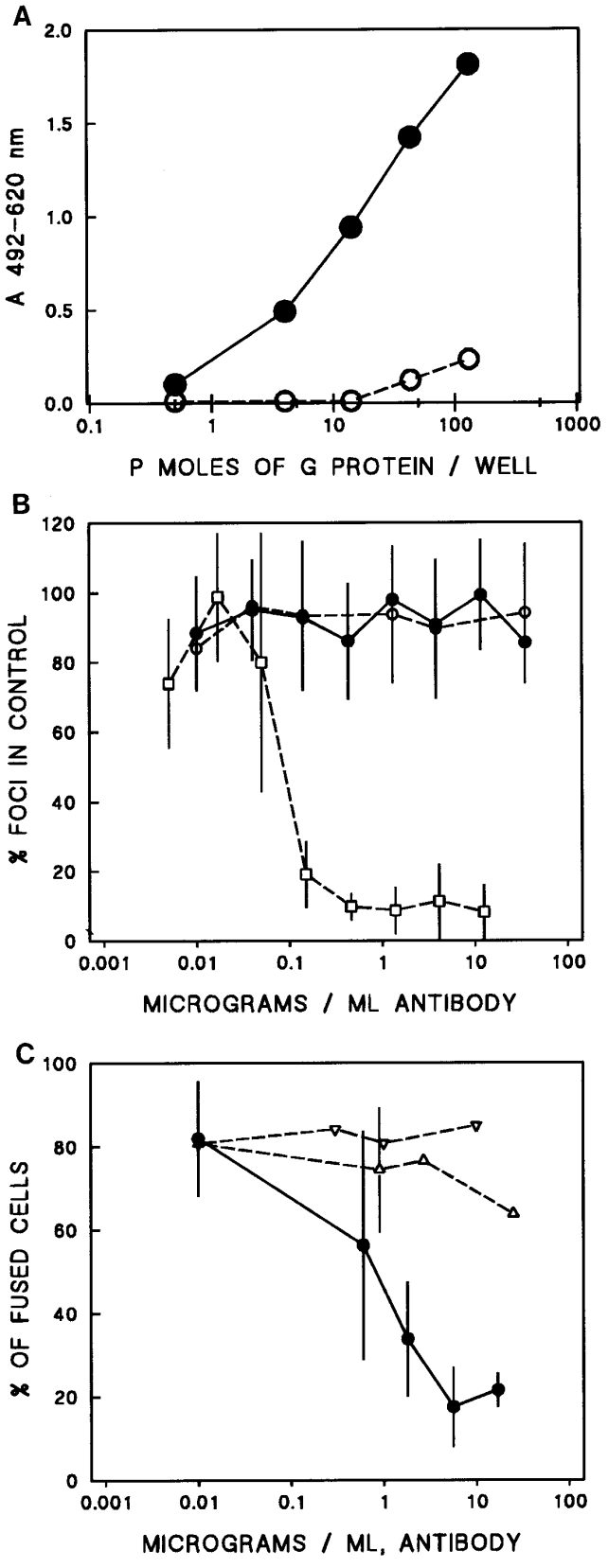


FIG. 5. Interactions of anti-p2 antibodies and VHSV. (A) Reaction of anti-p2 with several amounts of solid-phase-purified VHSV (expressed as its G content) was estimated by ELISA as indicated. (B) Neutralizing antibodies were estimated by reduction of DAB-positive foci by the neutralization/immunoperoxidase assay using 100 plaque forming units of VHSV. After overnight incubation and fixing, staining of the VHSV foci was with MAb 2C9 (anti-N), peroxidase-labeled rabbit anti-mouse IgG, and DAB. Means and standard deviations of duplicated wells are represented. (C) Inhibition of cell to cell VHSV-induced fusion from within by anti-p2 antibodies was estimated by first incubating EPC cell monolayers overnight at 14° with a multiplicity of infection of VHSV of 30. Next day, serial dilutions of antibodies in a pH 5.6 buffer at 14° were added. Fusion was estimated after 30 min at pH 5.6, 14°, and after fixing and staining. The percentage of fused cells in the absence of antibody controls was 82 ± 12. Means and standard deviations from 3 to 4 wells are represented. A representative result of at least three different experiments each is shown in A, B, and C. (●) anti-p2 at pH 5.6, (○) anti-p2 at pH 7.6, (□) anti-G MAb C10 at pH 7.6, (△) unspecific mice IgG_1 + IgG_{2a} at pH 5.6, (▽) anti-N MAb 2C9 at pH 5.6.

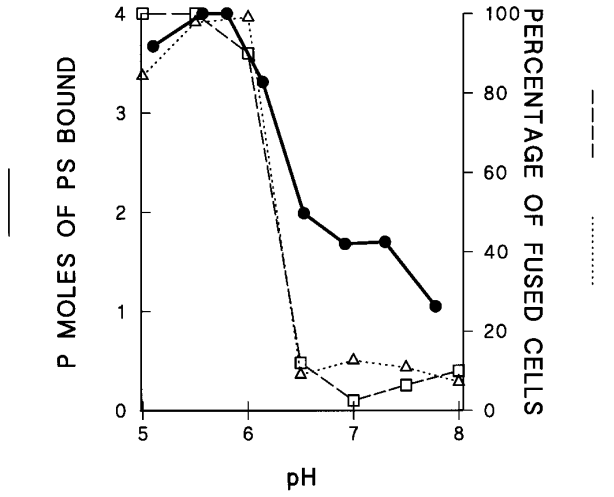


FIG. 6. pH dependence of PS binding to solid-phase VHSV and cell to cell fusion induced by VHSV infection. (●) PS binding to solid-phase VHSV (equivalent to 6 pmol of G/well) was during 4 hr at different pHs; (□) fusion from within was after overnight infection of EPC cell monolayers with 30 plaque forming units (PFU) of VHSV per cell and incubation during 30 min at 14° at different pHs; (△) fusion from without was after 1 hr at 4° incubation with 300 PFU of VHSV per cell, washing, and incubation during 30 min at 14° at different pHs.

than that of p106, they were still lower than the apparent specific activity of PS binding obtained by purified VHSV when expressed per picomole of G (Fig. 2 and Table 2). This difference could be due to the requirement of (a) other stretch(es) of the G protein sequence for maximal PS binding (as suggested by the existence of other PS-binding peptides in the pepscan shown in Fig. 1), (b) an intact G trimer structure, which could only be present in the purified virion or in Gs (Gs has a PS binding about 10-fold higher than that of denatured G), (c) collaboration of other viral proteins (Li *et al.,* 1993; Zakowsky *et al.,* 1981; Barge *et al.,* 1993), and/or (d) the presence of the viral membrane or of the viral membrane anchorage of the G protein. Since a total of three regions of newly defined a–d hydrophobic heptad repeats do exist in the G protein of VHSV (Table 3, aa 68–102, aa 288–319, and aa 377–400) (Coll, 1995b), some of them might be

TABLE 3

Amino-terminal Hydrophobic aa (in Bold) Heptad Repeats (Positions abcdefg) of the G of Rhabdoviruses

VHSV	68	FEDINKG LVSVPTR IIHLPLS VTSVSAV ASGHYLH	*R*VTY*R*	107
VHSVDK	68	FEDINKG LVSVPTK IIHLPLS VTSVSAV ASGHYLH	*R*VTY*R*	107
IHNGP	99	IHKV LYRTICS TGFFGGQ TIE	*K*ALVEM*K*	126
RABMOK	140	WLRT VTTTKES LLLISPS IVEMDIY	G*R*TLHSP	171
RABPV	140	WLRT VKTTKES LVIISPS VADLDPY	D*R*SLHS*R*	171
RHRBGD	140	WLRT VKTTKES LVIISPS VADLDPY	D*R*SLHS*R*	171
RABSAD	140	WLRT VKTTKES LVIISPS VADLDPY	D*R*SLHS*R*	171
RABHEP	140	WLRT VKTTKES LVIISPS VTDLDPY	D*K*SLHS*R*	171
RABLEP	140	WLRT VKTTKES LVIISPS VTDLDPY	D*K*SLHS*R*	171
RABCVS	140	WLRT VRTTKES LIIISPS VTDLDPY	D*K*SLHS*R*	171
VSVGPN08	134	TVTD AEAHIVT VTPHSVK VDEYTGE WID	P*H*FIGG*R*	168
VSVGPNJA	134	TVTD AEAHIVT VTPHSVK VDEYTGE WID	P*H*FIGG*R*	168
VSVGPN29	138	AEAHIIT VTPHSVK VDEYTGE WID	P*H*FLGG*R*	168
RHGPORS	134	TVTD AEAAIVQ VTPHHVL VDEYTGE WVD	SQFING*K*	167
RHGM	134	TVTD AEAVIVQ VTPHHVL VDEYTGE WVD	SQFING*K*	168
RHVSVGR	134	TVTD AEAVIVQ VTPHHVL VDEYTGE WVD	SQFING*K*	168

Note. The heptads are separated by space. Amino-terminal positions of the first and last aa are numbered by including the signal peptide. Positively charged aa in the carboxy-terminal position of the heptads are in italics. VHSV (Thiry *et al.,* 1991); VHSVDK (Lorenzen *et al.,* 1993); IHNGP (Koener *et al.,* 1987); rabies (Tordo *et al.,* 1993); VSVGPN08, VSVGPNJA, and VSVGPN29 (Nichol *et al.,* 1989); RHGPORS (Gallione and Rose, 1985); RHGM (Rose and Gallione, 1981); and RHVSVGR (Vandepol *et al.,* 1986). IHN, infectious hematopoietic necrosis; RAB, rabies; VSVGPN, vesicular stomatitis virus, New Jersey; RH, vesicular stomatitis virus, Indiana.

needed for maximal PS binding (for instance, p376, a 15-mer peptide which is partially inside of one of the heptad repeats of VHSV, also binds PS) (Fig. 1).

The p2 peptide (aa 82 to 109) was inside a region of five a–d hydrophobic aa heptad repeats (aa 68 to 102). Since the newly defined heptad repeats included hydrophobic aa W, H, and T, no coiled coil was predicted in this region by the use of current computer programs (Cohen and Parry, 1986; Lupas *et al.,* 1991), which only include F, Y, I, L, V, M, and A as hydrophobic aa. Thus, previous analyses of the VSV G sequence for the presence of a coiled-coil structure also failed to identify any predicted coiled coil.

However, because from aa 82 to 102 there is a putative α-helix with hydrophobic aa heptad repeats, this region could probably adopt the conformation of an amphipathic α-helix. An amphipathic α-helix followed by two positively charged aa (R) could interact with both the fatty acids and the two negative charges of a PS molecule. Experimental evidence (manuscript in preparation) shows that the physicochemical nature of the PS–p2 bond seems to be both hydrophobic (destroyed by detergents) and ionic (destroyed at pH $>$12.5, pH at which the charge of the R is about neutral). Hypothetically, the PS binding to solid-phase p2 could thus be explained by groups of p2 molecules with their hydrophobic sides facing either the solid phase or the fatty acid chains of the bound PS molecules and their charged sides facing the aqueous solvent interacting with the negative charges of the bound PS.

Although it still remains to be determined which stage of the entry process, binding and/or fusion, requires the phospholipid binding domain (for instance, mutagenesis evidence further implicating this domain would probably clarify these relationships), these findings are probably relevant to fish rhabdovirus infections. Thus, the whole sequence of p2 in VHSV 07.71 (Thiry *et al.,* 1991) was completely conserved in the other VHSV sequence reported to date (Lorenzen *et al.,* 1993). Whether or not this conserved domain and/or its nearby regions could be related to membrane fusion (Chambers *et al.,* 1990) induced by the G of VHSV has not yet been completely demonstrated. However, similar pH-dependence profiles were obtained by PS binding to purified VHSV and by VHSV G-mediated membrane fusion (Fig. 6). Furthermore, anti-p2 antibodies inhibited the fusion reaction (Fig. 5C), suggesting the existence of some correlation between both phenomena.

The preliminary search for p2-like regions (a–d new hydrophobic aa heptad repeats followed by 2 positively charged aa) in other rhabdoviruses showed that such sequences are present in all those studied, although some variations exist in the number of heptad repeats, in the length of separation of the positively charged aa, and in the nature of the positively charged aa involved. Whether or not these p2-like regions also bind PS and/or are involved in membrane fusion is unknown at present, but preliminary experiments have shown that the p2-like regions of IHNV, rabies virus, and VSV also bind PS (unpublished results). The fact that the VSV putative fusion peptide, aa 123–137 (Zhang and Ghosh, 1994) or the hydrophobically photolabeled fragment of VSV, aa 58–221 (Gaudin *et al.,* 1995) lie adjacent or around the putative VSV PS-binding region, aa 134–161 (Table 3), suggests that they are functionally related. A similar case

is found with the putative PS-binding region of the rabies virus, aa 140–168 (Table 3), which is found inside the hydrophobically photolabeled fragment aa 102–179 (Gaudin *et al.,* 1995).

We could speculate that, independent of a possible previous receptor-mediated specific interaction, an electrostatic interaction between the positively charged aa in p2-like regions and the negatively charged groups of PS and/or other phospholipids in the host membranes (Lenard, 1993; Schlegel *et al.,* 1983) could explain a first low-affinity binding of VSV or of VHSV to PS of the host cell membranes at physiological pH. This binding could be easily inhibited by PS and/or other phospholipids in the millimolar range at physiological pH (Bailey *et al.,* 1984; Conti *et al.,* 1988; Schlegel *et al.,* 1983; Superti *et al.,* 1984). Following internalization of the virus, the low pH could trigger the PS-binding region to be exposed (for instance, by adopting a bent α-helix conformation). Because of the amphipathic character (hydrophobic aa heptad repeats) of the now exposed α-helixes, they would also interact with the fatty acids of the closer PS molecules (White, 1992), resulting in a stronger interaction. A dual interaction of this kind would confirm previous observations that disclosed a requirement of the fatty acids for fusion but only a small requirement for the head group, in contrast to the requirement for binding, by using synthetic spin-labeled phospholipids (Yamada and Ohnishi, 1986). Whether or not the so-called fusion peptide is carried over by the putative low-pH-triggered amphipathic α-helix p2-like region to interact with other membrane phospholipids or whether the p2-like region is pulled into the membrane by the fusion peptide, is not known. It is also not clear whether the PS interaction would be essential for fusion or if it is part of a mechanism that enhances virus binding during fusion at the low pH (Konieczko *et al.,* 1994) and whether what is true for a rhabdovirus (VHSV) would be true for other (VSV).

The sequence variation in the p2 region of several VHSV strains or isolates could give some indications as to whether this region is important in defining species specificity and/or virulence. It will also be interesting to test whether salmonid neutralizing antibodies recognize this region in preference to others. On the other hand, it is also expected that the application of phospholipid binding to solid-phase synthetic peptides to the study of VHSV/phospholipid as well as other viral–phospholipid interactions could help to clarify some of the mechanisms of viral entry into the cells.

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